Endothelialization of Bypass Grafts Using Autologous Stem Cells

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Dedication

This thesis is dedicated to my family--my parents, Chong and Loan, my sisters, Kim and Abbie, and my brother, Scott, who have always been there for me throughout this journey and have encouraged me to believe in myself.

Abstract

Coronary artery disease is the most common type of cardiovascular disease and the leading cause of death in the United States. Coronary artery bypass grafting (CABG) is the standard of care but may be limited by the availability of suitable autologous grafts. Despite synthetic substitutes showing success in large-diameter bypass grafts, they are not suitable for replacing small-diameter vessels. Tissue-engineered vascular grafts (TEVG) can address limited availability of autologous vessel grafts. Endothelialization of luminal grafts can be done through pre-seeding with non-thrombogenic cells. Adipose-derived mesenchymal stem cells (ASCs) are good source of autologous stem cells for vascular differentiation, especially endothelial cells (ECs). In this work, we seeded ASCs onto fibroblast-remodeled fibrin tissues to create a hemocompatible TEVGs. We investigated the effects of shear stress, cyclic stretching, and simultaneous cyclic stretching and shear stress (resulted from pulsatile fluid flow) in differentiating ASCs towards ECs. Conditioned-ASCs aligned with the direction of flow but perpendicular to the direction of cyclic stretching. Although no detection of endothelial markers in conditioned-ASCs was observed, we found a reduction in platelet binding and an increased in nitric oxide production after exposure to biomechanical forces, indicating a potential utility of ASCs as non-thrombogenic luminal surface.

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Chapter 1. Hemocompatible tissue-engineered vascular grafts using adult mesenchymal stem cells

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1.1 Synopsis

Vascular tissue engineering can now produce compliant and durable vascular grafts to address limited supply of autologous vessel grafts for patients with coronary artery disease. Due to the demand for an anti-thrombogenic luminal surface, mesenchymal stem cells (MSCs) have been investigated for their potential to differentiate into an endothelial phenotype. This can be done through several types of chemical and biomechanical stimulation. Adipose-derived MSCs are of particular interest because they present an autologous source of sufficient MSCs to seed a monolayer onto the lumen of a typical coronary bypass graft. This review provides an overview of recent developments in endothelial differentiation methods of MSCs and main findings, as well as perspectives on future research.

Keywords: tissue-engineered vascular grafts, mesenchymal stem cells, endothelial cells, chemical factors, shear stress, cyclic stretching

1.2 Introduction

1.2.1 Cardiovascular disease and standard of care treatment

Cardiovascular disease is the leading cause of death globally, claiming more than 17.3 million deaths in 2013, expected to increase to more than 23.6 million by 2030. Coronary heart disease is the most common type of cardiovascular disease.[1] Despite advances in percutaneous coronary intervention and stent technology, coronary artery bypass grafting (CABG) is still performed, especially for complex lesions (high or intermediate SYNTAX scores).[2] There were 202,900 CAB grafts operated in the U.S. in 2012.[3]

Both arteries and veins have been used as autologous CAB grafts. The internal mammary artery (IMA) graft has been shown to have superior patency (10-year patency >90%)[4-6] with use of the left IMA yielding better survival rates.[7,8] Despite the superior patency rate of IMA, saphenous vein grafts are also commonly used for CABG patients[9] due to higher complications (e.g. mediastinitis) associated with removal of IMA.[10] About 50% of saphenous vein grafts are patent 10 years after CABG.[11] The failure of these grafts once patency is lost will require reoperation, but options may be limited by the availability of suitable autologous grafts. About ~30% of patients do not have suitable vein grafts (1979) due to vascular or harvested vein for prior vascular procedures.[12]

1.2.2 Tissue-engineered vascular grafts

To address the problems of limited autologous vessels for use in CABG, tissue-engineering approaches for small-diameter blood vessels have been developed using some combination of cells, scaffolds, and biochemicals. Applications of TEVG are also

potential solution to bypass grafts in hemodialysis and peripheral arterial disease (PAD). It is estimated that 19% of patients with end-stage renal disease use arteriovenous grafts[13] and PAD requires 45,000 bypass graft procedures yearly in the U.S.[14] The desired properties for tissue engineered vascular grafts (TEVG) emulating those of the native artery are sufficient mechanical strength, physiological compliance, durability, infection resistance, and hemocompatibility (anti-thrombogenicity).[15] One example is the fabrication of "tissue sheets" from smooth muscle cells (SMCs) and/or fibroblasts rolled into a fused tube that has shown high burst strength and good surgical handling.[16] Another example is production of a TEVG by entrapping dermal fibroblasts in a sacrificial fibrin gel tube, yielding tubes of cell-produced collagenous matrix with circumferential alignment as well as high burst strength,[17] which have been decellularized and successfully implanted into baboons as arteriovenous grafts for up to 6 months.[18] The same success was first demonstrated with decellularized TEVG made from SMCs seeded on a biodegradable synthetic polymer (polyglycolic acid) scaffold.[19] Although tissue engineering approach can produce the requisite mechanical properties, a strategy is needed to confer an anti-thrombogenic luminal surface to prevent thrombosis upon implantation.

Strategies to confer an anti-thrombogenic luminal surface can be classified into (1) chemical treatment of the surface to mitigate thrombosis and/or induce spontaneous endothelialization, and (2) seeding of the surface with cells that are anti-thrombogenic. These two approaches can also be combined. Surface modifications of the lumen have some disadvantages, such as short half-life of anti-thrombotic moieties[20] and lack of

endothelial selectivity of immobilization moieties.[21] Pre-seeding TEVG with conditioned non-thrombogenic cells holds the potential to improve hemocompatibility. This review will thus focus on the cell-based approach (2) within the last five years.

1.3 Role of endothelial cells in hemocompatibility

A thin layer (<0.2 um) of endothelial cells (ECs) acts as a dynamic barrier on the lumen of blood vessels that is responsive to hemodynamic forces and chemical stimuli in blood (Fig. 1).[22] They are crucial in regulating hemocompatibility by preventing adhesion and aggregation of platelets leading to thrombosis. Upon vascular injury, the endothelial layer is disrupted and subendothelial matrix is exposed to blood flow. Circulating platelets adhere to the exposed matrix through specific integrins. This leads to activation of the clotting cascade through thrombin production to convert soluble protein fibrinogen to insoluble fibrin, leading to thrombus formation.[23] When the endothelium is inflamed, ECs express elevated levels of adhesion molecules such as intercellular adhesion molecule-I (ICAM-1), vascular cell adhesion molecule-I (VCAM-1), E-selectin and P-selectin that can recruit platelets and leukocytes via strong adhesion.[24,25]

There are several endogenous mechanisms for ECs to prevent platelet adhesion. Glycocalyx, comprising glycoproteins and proteoglycans, is an interfacial layer between blood flow and the endothelial cell plasma membrane that regulates endothelial permeability and leukocyte interactions with ECs.[26] Studies have shown disrupting the glycocalyx layer leads to platelet adhesion to the endothelium; for example, Vink et al. damaged the glycocalyx with light dye-induced generation of oxygen-derived free radicals and found platelets and red blood cells adhered to the vascular wall.[27] ECs of

every blood vessel co-release two vasoactive hormones-nitric oxide (NO) and prostacyclin-that are inhibitors of platelet activation and vasoconstriction.[28] NO activates guanylyl cyclase to convert GTP to cGMP. Prostacyclin upregulates adenylate cyclase, yielding cAMP. Higher levels of cGMP and cAMP are anti-thrombotic.[29] Endothelial ecto-ADPase/CD39/NTPDase-1 is a cell surface enzyme that acts on the platelet releasate. When activated platelets release ADP from their granule that is responsible for platelet activation and recruitment, ecto-ADPase/CD39/NTPDase-1 removes ADP by converting it to AMP and subsequently to adenosine.[30] ECs can also promote fibrinolysis by secreting tissue plasminogen activator (tPA).[31] tPA activates plasminogen to plasmin, leading to degradation of fibrin. Endothelial dysfunction can thus shift the endothelium away from hemocompatibility and towards thrombosis.

1.4 Adult stem cells for vascular tissue engineering

Noting that (1) 20-30% of CABG cases are "urgent" (requiring surgery within 15 days),[32] (2) only use of an autologous cell source will ensure no immune response, and (3) the only autologous cell source that can provide enough cells to seed a CAB graft with a monolayer on a time scale relevant for urgent CABG patients with the potential to confer antithrombogenicity is adipose. This is because mesenchymal stem cells (MSCs) in the stromal vascular fraction are much more abundant as compared to bone marrow, and the volume of bone marrow that can be harvested is constrained for safety. Similar constraints apply to blood-derived sources of endothelial cells and progenitors. Thus, this review focuses on MSCs as a clinically-relevant cell source for urgent CABG patients. It is notable that whatever strategy is used to induce differentiation of MSCs *in vitro* will

need to yield an endothelial phenotype that is sustained (or even matured) post-implantation when exposed to the hemodynamic forces and chemical stimuli in blood experienced by a normal endothelium (Figure 1).

1.4.1 Mesenchymal stem cells

Stem cell-based therapies and tissue engineering have shown a potential for providing new solutions to cardiovascular diseases. The use of adult stem cells, specifically MSCs, for treating vascular disease, have gained momentum for autologous therapies due to their multipotency (differentiation into adipocytes, osteoblasts or chondrocytes) and relative ease of harvest from patients, such as from bone marrow (BM) or adipose.[33] They are also immunosuppressive because although they express Major Histocompatibility Complex Class I (MHC-I), they express relatively low levels of MHC-II (potent alloantigens) and can regulate the immune response pathway by soluble factor secretion and cell contact-dependent mechanisms.[34] Han et al. demonstrated MSCs suppressed the proliferation of CD4(þ) T cells by cell-cell contact *in vitro* and prevented secretion of various T cell cytokines.[35] Immunosuppressive mechanisms of these cells make them attractive for potential allogeneic therapies. A recent study from Krawiec et al.[36] constructed TEVG using xenogeneic MSCs isolated from human adipose and found the grafts were still robust and patent after 8 weeks of implant in a Lewis rat model with no overt immune response. In addition to their immunomodulatory property, importantly, MSCs have been shown to be able to differentiate towards an endothelial phenotype using various growth factors and/or biomechanical stimulation, summarized in Tables 1 and 2.

1.4.2 Chemical stimulation of MSCs

Differentiation of MSCs to ECs is possible via incubation with various chemical factors for an extended period. Vascular endothelial growth factor (VEGF) has been used to study angiogenesis; hence, it was the earliest used growth factor to induce MSC differentiation towards ECs. VEGF (50 ng/mL) has been shown to induce differentiation of human BM-derived MSCs (BM-MSCs) to an endothelial phenotype after 7 days. Endothelial marker (KDR, Flt-1, and vWF) expression was increased and capillary-like structures formed on a matrix by the differentiated MSCs.[37] Chen et al.[38] isolated human MSCs from umbilical cord (UC) Wharton's jelly and BM and cultured them in 100 ng/mL VEGF and 50 ng/mL epidermal growth factor (EGF) up to 12 days. Immunochemical analysis showed staining of endothelial-specific marker expression (Flk-1, vWF, and VE-cadherin), and LDL-uptake assay also detected uptake of Dil-Ac-LDL in both UC-MSCs and BMMSCs. Another angiogenic factor treatment, using 10 ng/mL VEGF and 5 ng/mL basic fibroblast growth factor (bFGF), was shown to differentiate murine embryonic MSCs, C3H/10T1/2, derived from C3H mouse for 9 days. RT-PCR and immunofluorescence staining revealed that differentiated MSCs acquired endothelial markers such as CD31, vWF Flk-1, Flt-1, VE-cadherin, Tie2, EphrinB2 and Vezf1 after 9 days.[39] Wang et al.[40] cultured rat BM-MSCs in 50 ng/mL VEGF and human BM-MSCs in 50 ng/mL VEGF and 10 ng/mL bFGF for 7 days. Differentiated MSCs expressed both Flt-1 and Flk-1 (Figure 2). When rat and human BMMSCs were cultured with Rho and ROCK inhibitors (C3 and Y-27632, respectively), Flt-1 and Flk-1 expression was downregulated. Sphingosine-1- phosphate (S1P) can also promote

endothelial differentiation of MSCs. S1P was investigated using mouse BM-MSCs and shown to improve endothelial differentiation compared to VEGF stimulation over 7 days, with higher protein expression of CD31 and VCAM-1.[41]

Other studies have used endothelial growth medium (EGM-2) containing VEGF, hydrocortisone, human bFGF, human insulin-like growth factor 1, ascorbic acid, human EGF, gentamicin-amphotericin-B, fetal bovine serum, and heparin combined with or without additional exogenous VEGF on differentiation of porcine BMMSCs. These MSCs cultured in EGM-2 with additional 50 ng/mL VEGF for 10 days showed the highest acetylated LDL uptake and vWF, VE-cadherin, and PECAM-1 expression compared to MSCs cultured in EGM-2 and in EBM (endothelial basal medium containing no endothelial growth factors).[42] Zhang et al.[43] cultured human MSCs derived from adipose tissue (AD-MSCs) on decellularized ovine carotid artery with or without 50 ng/mL VEGF for 7 and 14 days. They displayed positive staining for vWF and Flk-1 compared to the control group of AD-MSCs seeded onto tissue culture plastic (TCP), which was greatest when cultured with VEGF.

The advantages of culturing MSCs with growth factors relate to the relative simplicity of adding any desired growth factor to the culture medium. However, growth factors are expensive, entail cGMP production for clinical use, and are for all practical purposes impossible to maintain at a set concentration during extended culture.

1.4.3 Biomechanical stimulation of MSCs

Biomechanical forces, such as shear stress (SS) and cyclic stretching (CS) resulting from pulsatile blood flow (Figure 1), have been attempted to differentiate MSCs

towards ECs with or without combined growth factor treatment. Early use of SS to differentiate stem cells towards ECs was done by Wang et al.[44] They developed a parallel-plate flow chamber (PPFC) and applied constant SS of 15 dyn/cm^2 onto murine embryonic mesenchymal progenitor cell line (C3H/10T1/ 2) for 6 and 12 h. They observed an increased in mRNA expression of CD31 (162-fold and 757-fold, respectively) and vWF (97-fold and 108-fold, respectively) compared to a static control group. mRNA levels of SMCs, such as TGF-b, PDGF-B, and PDGFR, decreased by 52%, 85% and 99%, respectively. Constant SS of 15 dyn/cm^2 also enhanced acetylated LDL uptake and increased capillary-like tube formation by 3-fold after 12 h compared to static control cells. Similarly, Yamamoto et al.[45] explored the use of SS to induce Flk-1 positive embryonic stem cells into ECs and found constant SS of 5 dyn/cm^2 increased both mRNA and protein levels of Flk-1, VE-cadherin, PECAM-1 after 24e72 h. After 24 h, SS alone induced PECAM-1- positive cell sheets, with increased expression of PECAM-1 when combined with VEGF.

These early studies led to more investigation on biomechanical forces to differentiate MSCs towards vascular cells. Bai et al.[46] explored different magnitudes of constant SS with or without chemical stimulation on rat BM-MSCs. After 24 h of SS stimulation (10, 15, 20, and 25 dyn/cm^2), they observed mRNA expression of VEGFR-2 decreased at higher levels of SS. When SS of 15 dyn/cm^2 was combined with 50 ng/mL VEGF for 24 h, it yielded the highest level of VEGFR-2 and tPA compared to either SS or VEGF alone. Yuan et al.[47] found higher constant SS (20 dyn/cm^2) stimulation for 2 days followed by 5 days of static culture had stronger effects on elevating the EC markers

vWF, VE-cadherin, and CD31 as well as VEGF production in human BM-MSC compared to lower SS (2 dyn/cm^2) and static control. Unlike those studies, Zhang et al.[49] used an orbital shaker system to create SS rather than a PPFC. They cultured human AD-MSCs in EGM-2 and 50 ng/mL VEGF for 3 wk and then applied orbital SS to produce 12 dyn/cm^2 at the periphery of the wells for 48 h. Protein and mRNA levels of CD31 and vWF were both elevated. NO production and tPA expression that are responsible for anti-thrombosis were also increased. Using an orbital shaker to create SS is relatively simple but lacks the spatial uniformity and controllability of SS compared to a PPFC. Kim et al.[49] expanded constant SS studies to human BM-MSCs seeded onto electrospun poly(L-lactide-co-e-caprolactone) tubular scaffolds with diameter of 5 mm and they were cultured in EGM-2. They tested two differentiation methods: (1) steady SS of 2.5 dyn/cm^2 for 4 days and (2) steady SS of 2.5 dyn/cm^2 for 1 day followed by 3% CS at 2 Hz for 3 days then 5% CS at 2 Hz for 4 days. mRNA levels of vWF, CD31, VE-cadherin, and E-selectin were highest at day 1 and then decreased through day 4 with SS alone; aSMA expression was not affected by SS, but the expression of other SMC markers, calponin and caldesmon, increased during day 1 and returned to baseline by day 2. Interestingly, in Method (2), mRNA expression levels for all EC markers increased during the 4-day CS phase compared to the static group whereas SMC markers (aSMA, calponin and caldesmon) were not different from the static group. Studies like this conducted using cells seeded on the luminal surface of a compliant tube are the most relevant configuration with respect to TEVG application but introduce the complication

of simultaneous cell stretching if pulsatile flow is used, if the TEVG has near physiological compliance.

1.5 Unresolved questions

Tissue engineering has now successfully produced small-diameter vascular grafts with clinically-relevant mechanical properties. *In vivo* studies have demonstrated robust grafts and host-cell infiltration into the TEVG after implantation, including the capacity to grow with the recipient.[50] Spontaneous endothelialization of TEVG has been reported in preclinical studies.[17,19,50-52] However, CABG is usually performed in patients with a history of cardiovascular disease, which these preclinical studies did not replicate, and so there is no certainty there would be efficient spontaneous endothelialization in CABG patients without prolonged anti-coagulation therapy, if ever. Thus, a clinically relevant strategy to ensure a non-thrombogenic luminal surface is still in demand, and the studies summarized above suggest AD-MSCs may be a viable strategy when time is of the essence, as for urgent CABG patients, and does not allow for needed cell expansion from other autologous cell sources (e.g. primary endothelial cells from a vessel biopsy, BM-MSCs, and inducible pluripotent stem cells (iPSCs), which may present other challenges as well, such as efficiency of transduction and safety using retroviral integration).[53]

The underlying mechanism governing endothelial differentiation of MSCs is still poorly understood even under a relatively simple environment such as culture with VEGF on TCP, not to mention the more clinically relevant but complex cell culture environment when seeded on a TEVG exposed to SS plus CS conditioning and endothelial growth factors. Reports of endothelial differentiation in MSCs tend to correlate with inhibition of

SMC differentiation but it remains unclear what pathways are involved in the regulation of differentiation.[54,55] One possible key pathway is through Rho/ myocardin-related transcription factor-A (MRTF-A) signaling. Wang et al.[40] studied the mechanism related to VEGF-induced MSC differentiation and found that VEGF induced Rho/ROCK that promoted MRTF-A signaling pathway, consistent with an increase in EC markers. MRTF-A upregulated Cyr61/CCN1 gene transcription that has pro-angiogenic activities in endothelial cells. Zhang et al.[56] further investigated the role of Rho/MRTF-A in VEGF-induced angiogenesis by differentiated MSCs. Rho/MRTF-A upregulating the expression of integrins a1, a5, and b1 was observed during angiogenesis. Both studies only investigated one common growth factor, VEGF, and did not address whether SMC markers decreased or not and through what signaling pathways. More studies are needed to elucidate the transcriptional mechanism of endothelial differentiation of MSCs to enable improved control and optimization *in vitro*.

As summarized in this review, combining growth factors with SS has been shown to yield greater effects on endothelial differentiation of MSCs than growth factors alone.[46,48] However, there are mixed results about the beneficial combination of SS and CS. Kim et al.[49] demonstrated the complementary effects of constant SS (1 dyn/cm^2) for 1 day followed by CS (3% then 5% strain, 2 Hz) for 7 days on endothelial marker expression with decreased expression of SMC markers. In contrast, Shojaei et al.[57] found that CS (10% strain, 1 Hz) for 1 day decreased expression levels of Flk-1 and vWF but not VE-cadherin, while pulsatile SS (0-2.5 dyn/cm^2) for 1 day elevated their expression; simultaneous pulsatile SS + CS for 1 day had minimal effects except on VE-cadherin.

These different results from combining SS and CS could be due to different CS magnitude, differentiation duration, and sequential vs. simultaneous application of SS and CS. Again, further studies are needed to elucidate the mechanism of endothelial differentiation of MSCs under SS alone and SS combined with CS, which will serve to optimize *in vitro* differentiation strategies and potentially ensure stability of an endothelial phenotype post-implantation where combined pulsatile SS + CS is perpetual. Understanding how cellular deformation resulting from SS and CS regulate differentiation may lead to improved biomechanical conditioning strategies.[58-60]

1.6 Conclusion/outlook

Although evidence indicates MSCs can differentiate into an endothelial phenotype, it is not yet proven that these cells can prevent thrombosis *in vivo* long-term in a pivotal preclinical model. Hashi et al.[61] showed BMMSCs seeded in nanofibrous vascular grafts possessed an anti-thrombogenic property both *in vitro* and up to 60 days in a rat model, so there is cause for optimism. In conclusion, AD-MSCs are an attractive autologous cell source and capable of differentiation to an endothelial phenotype to potentially provide a hemocompatible vascular graft generally and especially for urgent CABG patients.

1.7 Figures

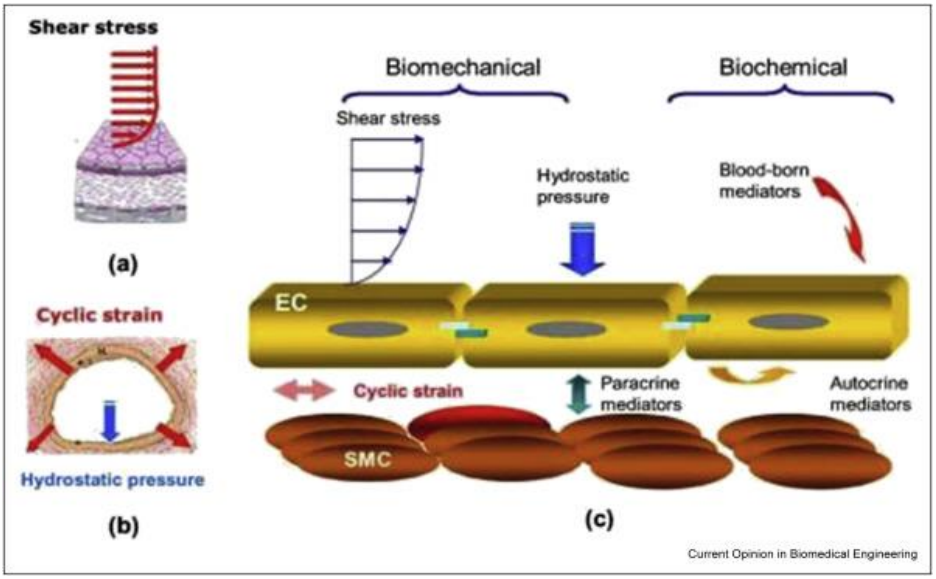

Figure 1-1. Schematic diagram of hemodynamic forces acting on endothelial cells (EC) and smooth muscle cells (SMC) in the blood vessel wall.

(A) Fluid shear stress, the tangential frictional force by virtue of blood viscosity, acts on ECs. (B) Cyclic strain exerts a circumferential stretch on arterial wall in response to cardiac contraction. Hydrostatic pressure acts perpendicularly on ECs. (C) ECs are constantly exposed to both biomechanical and biochemical stimuli, which modulate endothelial functional phenotype. The biochemical stimuli include hormones, growth factors, cytokines, and bacterial products that can be delivered via the blood or via autocrine or paracrine mechanisms. Reproduced without alteration from Ref. [22].

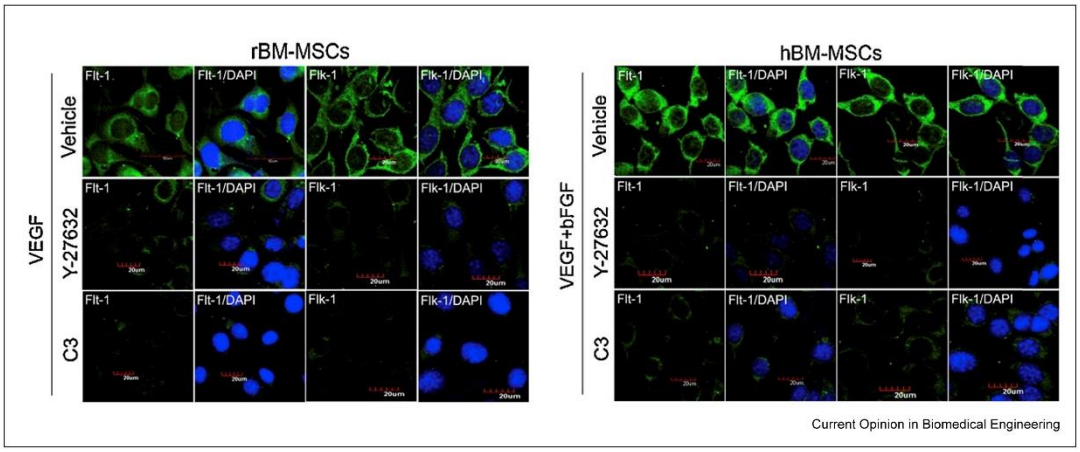

Figure 1-2. VEGF induced MSC differentiation to endothelial cells by Rho/ROCK signal pathway.

The expression of endothelial markers Flt-1 and Flk-1 was tested by immunofluorescent staining. Scalebar = 20 mm. Reproduced with modification from Ref. [40].

1.8 Tables

Reference	Cell type	Growth factors	Time points
Oswald et al. (2004)	Human BM-MSCs	50 ng/mL VEGF	7 d
Chen et al. (2009)	Human UC-MSCs Human BM-MSCs	100 ng/mL VEGF 50 ng/mL EGF	12 d
Wang et al. (2010)	Murine embryonic MSCs	10 ng/mL VEGF 5 ng/mL bFGF	9 d
Wang et al. (2013)	Rat BM-MSCs Human BM-MSCs	50 ng/mL VEGF 10 ng/mL bFGF	7 d
Lu et al. (2015)	Mouse BM-MSCs	1 μmol/L S1P 50 ng/mL VEGF	7 d
Pankajakshan et al. (2013)	Porcine BM-MSCs	EGM-2 50 ng/mL VEGF	10 d
Zhang et al. (2016)	Human AD-MSCs	50 ng/mL VEGF	7–14 d

Table 1-1. Chemical stimulation of MSC differentiation to an endothelial phenotype.

Reference	Cell type	Growth factors	Mechanical forces	Time points
Wang et al. (2005)	Murine embryonic mesenchymal progenitor cell	Not tested	Steady SS of 15 dyn/cm^2 (PPFC system)	6–12 h
Yamamoto et al. (2005)	Mouse Flk-1 positive embryonic stem cells	Not tested	Steady SS of 5 dyn/cm^2 (PPFC system)	24–72 h
Bai et al. (2010)	Rat BM-MSCs	50 ng/mL VEGF	Steady SS of 10–25 dyn/cm^2 (PPFC system)	7 d (VEGF static culture) 12–48 h (SS)
Yuan et al. (2013)	Human BM-MSCs	Not tested	Steady SS of 2 and 20 dyn/cm^2 (PPFC system)	2 d (SS) 5 d (static culture)
Zhang et al. (2011)	Human AD-MSCs	EGM-2 50 ng/mL VEGF	Orbital SS of 12 dyn/cm^2 at the periphery	3 wk (EGM-2 + VEGF static culture) 48 h (SS)
Kim et al. (2016)	Human BM-MSCs	EGM-2	Tubular steady SS of 2.5 dyn/cm^2 CS (2 Hz) at 3% and 5%	Step 1: 4 d (SS) Step 2: 1 d (SS) + 3 d (3%CS) + 4 d (5%CS)

Table 1-2. Biomechanical stimulation of MSC differentiation to an endothelial phenotype.

Chapter 2. Shear conditioning of adipose stem cells for reduced platelet binding to engineered vascular grafts

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2.1 Synopsis

Conferring antithrombogenicity to tissue-engineered vascular grafts remains a major challenge, especially for urgent bypass grafting that excludes approaches based on expanding autologous endothelial cells (ECs) that requires weeks of cell culture. Adipose-derived stem cells (ASCs) are available from most patients in sufficient number for coronary bypass graft seeding and may be effective as allogeneic cells. We thus compared the adhesion and platelet binding of human ASCs that were shear conditioned with constant and pulsatile shear stress (SS) after seeding the cells on a biologically engineered matrix suitable for arterial grafts. A monolayer of cells was maintained up to 15 dyn/cm^2 constant SS and up to 15 dyn/cm^2 mean pulsatile SS for 6 days of shear flow. Platelet binding was reduced from 83% to 6% of surface area and nitric oxide production was increased 23-fold with 7.5–15 dyn/cm^2 constant SS, but not pulsatile SS, relative to cells cultured statically on the matrix for 6 days. The reduction in platelet binding varied from no reduction to maximum reduction over a constant shear range of ~2 to 4 dyn/cm^2, respectively. Collectively, the study supports the potential use of ASCs to seed the luminal surface of a vascular graft made from this biologically engineered matrix to confer an antithrombogenic surface during the development of an endothelium from the seeded cells or the surrounding blood and tissue.

2.2 Introduction

Coronary artery disease is the most common cardiovascular disease in the United States and accounts for 1 in 7 deaths.[1] Although advances in percutaneous coronary interventions and stent technology have been introduced to treat this disease with the advantage of a noninvasive procedure, coronary artery bypass grafting (CABG) is often still the preferred alternative, especially for left main coronary artery disease,[62-64] with 434,255 CAB graft implants into 190,695 patients in 2012 (HCUP data).

Graft selection for CABG is crucial to the success of long-term patency. Both arteries and veins have been used as autologous CAB grafts, primarily internal mammary artery and saphenous vein. The failure of saphenous vein grafts (e.g., about 50% of saphenous vein grafts are patent 10 years after CABG[11]) requires reoperation but may be limited by the availability of suitable autologous vessel; about 30% of patients do not have suitable vein grafts due to harvested vein for prior vascular procedures.[12] Also, harvesting saphenous grafts requires a second surgical procedure, which may not be suitable for certain patients with medical constraints, and any autologous vessel graft entails second site morbidity.

Tissue-engineered vascular grafts (TEVG) have emerged to overcome many of the major limitations of comorbidities, limited availability of autologous vessel harvest, and frequent thrombogenicity in small-diameter synthetic grafts. The success of TEVG depends on the ability to emulate mechanical properties of the native vessels with an organized extracellular matrix that possesses a confluent and functional endothelium to resist thrombosis in vivo. Because a functional endothelium is the only proven recourse

for long-term thrombus prevention, achieving one on the luminal surface of a TEVG is crucial for long-term patency without sustained anticoagulant therapy. Although spontaneous endothelialization of our TEVG occurs in preclinical studies with healthy animals over several months depending on anatomical location,[17,50,65-67] if it cannot be realized in patients over acceptable time frames, even with treatment of the luminal surface with capture molecules such as SDF1,[68-70] then seeding EC onto the luminal surface of the TEVG is one potential option.

The substantial immunogenicity of EC necessitates an autologous source at present. While blood outgrowth ECs are an attractive autologous source for elective CABG patients who can wait the weeks to months required for their isolation and expansion,[71] a clinically suitable source of primary EC is lacking in the case of urgent CABG (needed within 48 h to 15 days),[32] which comprises between 20% and 30% of CABG cases.[72-74]

Mesenchymal stem cells (MSCs) have been investigated for the treatment of vascular diseases since they can differentiate into EC.[37,42,48] MSC can be found in human bone marrow,[75] adipose tissue,[76] or umbilical cord blood.[77] They have immunomodulatory capacity and immunosuppressive properties due to their ability to regulate the immune response system through direct cell-to-cell interactions and secretion of soluble factors[33] and have demonstrated efficacy in some studies as allogeneic cells.[78,79] Recently, Krawiec et al.[36] fabricated TEVG using xenogeneic MSCs (derived from human adipose tissue from healthy donors) and showed robust and patent grafts

lined with von Willebrand factor (vWF)-positive cells in a Lewis rat model after 8 weeks of implantation as well as no overt immune response.[36]

Isolating MSC from adipose tissue (termed adipose-derived stem cells or ASCs) is clinically feasible, less invasive compared to isolation of MSC from bone marrow (termed BM-MSC), and robust with respect to advanced patient age and comorbidity[48,80] except for diabetes.[81] Adipose tissue harvest also yields much more abundant MSC (1–10%)[82] than bone marrow harvest (0.001–0.01%),[83] which makes ASC a very attractive source for TEVG seeding before implantation if autologous sourcing proves necessary.

To use ASC for TEVG, efficient and reliable strategies to differentiate ASC to EC are needed as well as avoidance of differentiation into smooth muscle cells, osteoblasts, and chondrocytes. It was shown that chemical factors, such as vascular endothelial growth factor (VEGF), stimulated ASC to express EC markers.[48] Potent but less explored mediators of ASC differentiation are shear stress (SS) and cyclic stretching, which are relevant for CABG because of immediate exposure of the seeded cells to pulsatile blood flow. Constant SS alone has been reported to induce EC differentiation of ASC[48] as well as in combination with VEGF,[84] consistent with similar studies using BM-MSC.[46] However, no previous studies have assessed the effects of pulsatile SS on ASC differentiation nor has there been a direct demonstration of functional antithrombogenicity—as measured by reduced platelet binding in vitro—by shear-conditioned ASC. Pulsatile SS is of particular interest since this would be the shear regimen experienced immediately post implantation of an ASC-seeded TEVG in the arterial system.

In this study, we compared the adhesion and platelet binding of human ASC shear-conditioned with constant and pulsatile SS after seeding the cells on a biologically engineered matrix that we previously used in preclinical studies with[85] and without[17] pre-seeded ECs. In addition, the SS range that yields reduced platelet binding was determined using a parallel plate flow chamber (PPFC) based on Usami et al.,[86] wherein SS decreases linearly with axial position along the chamber. We also assessed nitric oxide (NO) production, vascular endothelial (VE)-cadherin, and surface expression of heparan sulfate proteoglycan (HSPG2) as a possible explanation for reduction in platelet binding of the shear-conditioned ASC.

2.3 Materials and methods

2.3.1 Isolation and culture of ASC

Human ASCs (Lonza) were plated at 5000 cells/cm2 and cultured in expansion medium (ADSC-GM, Lonza), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C, 5% CO_2. Medium was replaced every 3 to 4 days. After ASCs reached near confluency (~90%), cells were passaged for subsequent experiments and used at passage 5.

2.3.2 TEVG preparation

TEVG from fibroblast-remodeled fibrin gel was prepared using our established protocols.[87] Thrombin (1.1 U/mL) and calcium chloride (5.0 mM) were added to fibrinogen (4 mg/mL) solution with suspended human dermal fibroblasts (Clonetics) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline. After gelation, the samples were removed from the tubular mold and cultured on the 4

mm diameter glass mandrel in Dulbecco's modification of Eagle's medium (DMEM; Cellgro) containing 10% fetal bovine serum (FBS; EMD Millipore), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mg/mL insulin, and 50 mg/mL ascorbic acid. Medium was changed three times per week for 2 to 3 weeks to allow for initial gel compaction and associated circumferential alignment of the cell-produced matrix. Following this static culture, the grafts were mounted in pulsed flow-stretch bioreactors at 0.5 Hz pulse frequency and constant strain amplitude circumferential strain in the range 3–7% for 5–7 weeks.[87]

After bioreactor maturation of the grafts, they were decellularized as previously described.[50] The constructs were rinsed with phosphate-buffered saline (PBS) for 10 min at room temperature with gentle rocking, followed by 1% sodium dodecyl sulfate (SDS; Sigma) for 6 h. Subsequently, they were rinsed with 1% Triton X-100 (Sigma) for 30 min, followed by 1 week of PBS rinses. A final treatment with deoxyribonuclease enzyme (DNase; Worthington Biochemical) in DMEM/10% FBS at 37C, 5% CO2 for overnight incubation was used to maximize removal of residual DNA. Grafts were then washed with PBS for 3 to 4 days to continue removing any residual SDS/Triton/DNase/cellular components and stored in PBS at 4C until use.

2.3.3 SS conditioning

The decellularized grafts were cut longitudinally into 3 x 0.5 cm rectangles and embedded in 3% agarose in polycarbonate flow chamber inserts to expose the luminal surface for ASC seeding.[88] The biologically engineered matrix was pretreated with 50 mg/mL fibronectin (Sigma, F1141), and then, ASCs were seeded as a monolayer from a

suspension (1.5 x 10^6 cells/mL) onto the mounted matrix and incubated for 24 h. After the static culture period, a subset of the samples was subjected to targeted SS for 6 days in our custom PPFCs with an inlet width of 1 cm, length of 3 cm, and height of 0.05 cm. The varying width of the chamber was adapted from Usami et al.'s study[86] to apply a linear range of SS. Endothelial growth medium (EGM-2; Lonza) was used as a source of endothelial differentiation factors, including VEGF, human basic fibroblast growth factor, and human epithelial growth factor. A pulse dampener was placed right after the peristaltic pump to create constant SS or removed to create pulsatile SS at 0.5 Hz. The Womersley number (Wo) is a dimensionless number measuring the ratio of inertial forces to viscous forces.

$$W_o = h\sqrt{\frac{\omega\rho}{\mu}}$$

where h=chamber gap height, ω=wave radian frequency, ρ=fluid density, μ=fluid viscosity. It characterizes the unsteady flow due to an unsteady pressure gradient. If $W_o < 1$, the velocity profile is parabolic and in phase with the pressure gradient ("quasi-steady"). If $W_o > 1$, flow is not parabolic and velocity profile is out of phase with the pressure gradient. In our experiments, $W_o = 0.35$ and so the flow was quasi-steady, so for both constant and pulsatile flow experiments, the result for constant slit-flow of an incompressible Newtonian fluid could be applied to calculate the instantaneous wall

shear stress, τ_{wall}, for instantaneous volumetric flow rate, Q(t), at each axial position, x, with width w(x):[37]

$$\boldsymbol{\tau_{wall}(t,x) = \frac{6 * \mu * Q(t)}{w(x) * h^2}}$$

2.3.4 Platelet adhesion assay

Human platelets (Red Cross) suspended in platelet-rich plasma were centrifuged at 1300 g for 10 min to yield the platelet pellets. Pellets were suspended in ‘‘acid citrate dextrose’’ buffer (100 mM trisodium citrate, 10 mM citric acid, and 136 mM glucose, pH 6.5). The platelets were centrifuged again at 1300 g for 10 min and suspended in Tyrode’s solution (134 mM sodium chloride, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, 0.34 mM sodium phosphate monobasic, 1 mM magnesium chloride, 10 mM HEPES, and 5.5 mM glucose, pH 7.4). Samples were incubated in suspended platelets for 2 h at 37C. After incubation, they were rinsed with Tyrode’s solution two to three times. The samples were fixed with 1% paraformaldehyde for 30 min at 4°C followed by 3 x 5 min PBS rinses.

2.3.5 Immunostaining

The samples were permeabilized by 0.1% Triton X-100 (Sigma) and blocked with 5% normal donkey serum at room temperature. They were incubated with primary antibodies CD41 for glycoprotein IIB/IIIA (Abcam, ab11024, 1:400), HSPG2 (Abcam, ab26265, 1:100), and VE-cadherin (Abcam, ab33168, 1:200) overnight at 4C. Species-matched secondary (Cy3- or Cy5-conjugated, 1:200) antibody (Jackson Immunoresearch)

and Alexa Fluor 488 phalloidin (Life Technologies, 1:400) were added to the samples. Cell nuclei were stained with Hoechst 33342 (Invitrogen, H3570, 1:10,000).

2.3.6 Nitric oxide assay

Medium samples were collected from each of the flow conditions and frozen at -80C. Using a Fluorometric Assay Kit (Abcam, ab65327), nitrite in the samples was converted to nitrate during 3-h incubation. Enhancer, DAN probe, and sodium hydroxide were added to the converted nitrate and was measured at 360/450 nm (excitation/emission).

2.3.7 Alignment of ASC

Cell alignment on the matrix was evaluated post-flow by phalloidin staining and analyzed using an ImageJ orientation plugin[89] to determine the orientation angle relative to the flow (SS) direction.

2.3.8 Statistical analysis

Results are expressed as mean ± standard deviation with at least three repeated experiments (indicated by ‘‘n’’). Statistical significances were evaluated by one-way analysis of variance for multiple groups or one sample t-test (for cell orientation), compared to a theoretical value of 1, with a probability value of $p < 0.05$ considered statistically significant.

2.4 Results

2.4.1 Linear SS parallel plate flow chamber design validation

Assessing different levels of uniform SS for endothelial differentiation of ASC in vitro in a conventional PPFC would be time consuming and require many samples to be

run in separate experiments. The PPFC of Usami et al.,[86] wherein SS varies linearly with axial position in a prescribed region, was modified to study the effects of selected ranges of constant laminar SS on ASC pre-seeded on biologically engineered matrix embedded in agarose within an insert that mounts flush with the flow surface.[88] Figure 2-1A shows a top view of the chamber and the contour of the sidewalls needed to achieve a region of linear dependence of SS (a conventional PPFC uses sidewalls that have a constant separate distance along the chamber axis). The resulting SS profile along the chamber axis for constant laminar flow from left to right for a fixed volumetric flow rate computed using COMSOL Multiphysics is shown in Figure 2-1B. The SS is highest near the inlet and then decreases linearly with distance from 15 to 7.5 dyn/cm^2 over a 3 cm length in the central region (where a rectangular sample is located) until it becomes nonlinear with distance toward the outlet. The decrease in SS by a factor of two occurs for any chosen inlet flow rate given the chamber design, a feature that was also used in this study. As shown in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/tea), at any axial position in the boxed region, the predicted SS is uniform over ~90% of the width, and only cells within that domain were assessed.

2.4.2 ASC retention and platelet binding post-shear conditioning

ASCs seeded onto slabs of the biologically engineered matrix were mounted flush onto one plate of the PPFC, cultured statically for 1 day, and then subjected to constant or pulsatile SS in a ramp over 2 days to a target value and held for 4 days. Samples were harvested and assayed for retention via staining with Hoechst and for platelet binding via immunostaining for glycoprotein IIB/IIIA. Figure 2-2A shows that the unseeded matrix

led to extensive platelet binding, as expected, given it is composed of extracellular matrix (Supplementary Figure S2), including collagen, fibronectin, and remnant fibrin,[17,85] known to bind platelets. It was found that platelet binding was reduced following ASC for static culture the entire 7 days (Figure 2-2B), but the reduction was greater for ASC cultured for 6 days under constant SS for the range 7.5–15 dyn/cm^2, during which time a monolayer of ASC was maintained (Figure 2-2C, 2-2D). There was no dependence on the magnitude of constant SS over the range 7.5–15 dyn/cm^2 (Supplementary Fig. S3), over which a monolayer of ASC was maintained (Supplementary Fig. S4), and there was no difference in the reduction using constant and pulsatile SS of mean 15 dyn/cm2 (Fig. 2-2E; see Supplementary Fig. S5 for pulsatile waveform). Interestingly, there was a graded reduction in platelet binding over the range 2 to 4 dyn/cm^2 of constant SS (Fig. 2-3A) with a difference across this range (Fig. 2-3B), over which a monolayer of ASC was again maintained (Fig. 2-3C). The platelet binding measured at 2 dyn/cm^2 was approximately the value measured for static incubation (Fig. 2-2E) and the value measured at 4 dyn/cm^2 approached that measured in the range 7.5–15 dyn/cm^2 (Supplementary Fig. S3), indicating 4 dyn/cm^2 of ASC shear conditioning to be an approximate threshold for maximal reduction of platelet binding to the ASC.

2.4.3 ASC production of NO post-shear conditioning

NO production is a hallmark of the endothelium, and NO is well known to be a platelet inhibitor (as well as vasodilator), so its production in these experiments was measured. Medium collected from the chambers for the same three ASC-seeding groups presented in Figure 2-2E was analyzed for NO via conversion of its natural degradation

product nitrite to nitrate. Figure 2-4 shows that there was dramatic 23- fold increase of NO produced in the cases of constant shear conditioning relative to static culture for the entire 7-day incubation. Interestingly, there was also an NO production increase resulting from constant shear relative to pulsatile shear. There was no apparent upregulation of HSPG2 or VE-cadherin resulting from shear conditioning based on immunostaining (Supplementary Fig. S6).

2.4.4 ASC orientation post-shear conditioning

ASC orientation was measured as from F-actin-stained stress fibers for the same ASC-seeding groups presented in Figure 2E is shown in Figure 5. Only the static culture group had a value of $<\cos\theta>$ different from the flow direction, with both constant and pulsatile shear conditioning leading to ASC alignment with the flow direction ($<\cos\theta>=1$). The value of $<\cos\theta>$ for the the static culture group was consistent with random orientation ($<\cos\theta>=0.5$).

2.5 Discussion

The motivation for this study was to ascertain whether short-term shear flow conditioning in vitro could be used to drive ASC differentiation toward an antithrombogenic phenotype post-seeding on biologically engineered matrix as a strategy to confer rapid hemocompatibility for urgent CABG applications, using tubes of this matrix as a TEVG. Previous studies have indicated that shear conditioning of ASC on various synthetic substrata has resulted in the expression of endothelial markers and function consistent with increased antithrombogenicity, namely NO production.[48] However, a more definitive assessment of antithrombogenicity has not been reported, and

no assessment of ASC on a biologically engineered matrix has been reported. Our TEVG—a decellularized tissue tube grown in vitro from dermal fibroblasts in a sacrificial fibrin scaffold—has been shown to yield physiological burst pressure and stiffness[17,87] and confer excellent retention of ECs both in vitro and in vivo.[85,88] It also endothelialized spontaneously over time in preclinical studies using healthy animals.[7-9,17,50,65] It was thus considered a relevant matrix to use for these experiments performed to assess whether short-term shear flow conditioning could result in decreased platelet binding to the seeded ASC as well as increased NO production, and whether any such effects depended on the shear flow being constant or pulsatile.

The linear shear flow PPFC conceived by Usami et al.[86] was very advantageous for these studies. Not only did it allow for assessment of how ASC retention on the matrix varied with SS without repeated experimentation as would be required using a conventional constant shear flow PPFC but it also allowed for determination of a threshold constant SS, above which a further reduction in platelet binding did not result. A previous study[90] experimentally validated the linear shear flow predicted by Usami et al.[86] and used computational fluid dynamics to demonstrate the quasi-steady solution (where the surface SS is given by the steady-state solution at the instantaneous flow rate, to good approximation) for sufficiently small (<1) Womersley number, in their case 0.75 and in our case 0.35.

The reduction in platelet binding over the range 2 to 4 dyn/cm^2 cannot be attributed to loss of an ASC monolayer and exposure of the underlying matrix, as a monolayer was observed with Hoechst staining, and indicates a differentiation toward an

antithrombogenic phenotype. No further reduction of platelet binding was observed beyond a constant SS of ~4 dyn/cm^2, indicating a threshold for differentiation. Surprisingly, there was no benefit of pulsatile shear conditioning over constant shear comparable to the time-averaged value, at least for the waveform used. These observations provide some gross clues to the biophysical mechanism of shear mechanotransduction.[91] For example, if nuclear deformation determines the ASC differentiation leading to the observed platelet binding inhibition, these results suggest that either the deformations resulting from the steady and pulsatile waveform used are no different, or, more likely, the resulting differences are inconsequential. Further clues might be obtained from testing other frequencies and amplitudes that can be related to dynamics of second messenger dynamics and cytoskeletal/nuclear strains, respectively.

Low-magnitude laminar SS (e.g., 0.3–2.7 dyn/cm^2) has been shown to induce BM-MSC/ASC toward osteogenic cells rather than EC,[93-94] whereas laminar arterial SS (e.g., 15 or 20 dyn/cm^2) has been reported to increase messenger RNA (mRNA) and protein expression of endothelial markers in BM-MSC.[47,95] These reports are consistent with our finding that the reduction in platelet binding became more pronounced as laminar SS increased to 4 dyn/cm^2 and was independent of SS magnitude over the range of 7.5–15 dyn/cm^2. The relatively low value of the threshold in our system for apparent endothelial differentiation (i.e., 4 vs. 15 or 20 dyn/cm^2) may reflect a difference in the outcome measure used (platelet binding reduction vs. EC marker expression), environmental conditions (biological matrix vs. synthetic substrate), or MSC type (ASC vs. BM-MSC).

Endothelial NO is well known to inhibit platelet activation, adhesion, and aggregation, and a deficiency of bioactive NO is associated with arterial thrombosis.[96] The marked 23-fold increase in NO produced by the ASC after constant shear conditioning relative to static culture may explain the observed reduction in platelet binding and is consistent with ASC differentiation toward an EC phenotype, although there are other endothelial mechanisms platelet binding inhibition that might also contribute to this reduction involving, for example, prostacyclin and HSPG2. The 23-fold increase we observed was substantially greater than the 3.6-fold increase reported by Zhang et al.,[48] but that can be attributed to any number of differences in the study conditions, including shear duration (2 vs. 6 days), SS platform (orbital vs. laminar), and substrate (decellularized human saphenous vein precoated with fibronectin vs. biologically engineered matrix precoated with fibronectin).

While elevated NO production is consistent with endothelial differentiation, we did not detect immunostaining for VE-cadherin and HSPG2, which was shown indirectly using heparinase to be the molecular mechanism leading to a reduction of platelet binding in shear-conditioned BM-MSC isolated from bone marrow.[61] Thus, despite the increased NO production and reduced platelet binding, whether the shear-conditioned ASCs were differentiating toward an endothelial phenotype in this study is an open question. Although Zhang et al.[48] demonstrated that SS increased NO production and upregulated mRNA levels of both VEcadherin and vWF in ASC, Bassaneze et al.[97] did not detect upregulation of any endothelial mRNA levels (e.g., vWF) in ASC after exposure to SS of 10 dyn/cm^2 for 96 h, even though NO production was increased. These different

responses of ASC to SS exposure could be due to different substrates and different magnitude and duration of SS.

Cell alignment with flow under constant and pulsatile shear flow in the normal arterial range is a hallmark of ECs seeded on a variety of substrates and has also been reported for ASC after 2 days of constant shear conditioning.[48] Our observation of ASC alignment with flow after 6 days of both constant and pulsatile shear conditioning is consistent with these results and with ASC differentiation toward an EC phenotype.

Any number of substrate properties can influence cell differentiation, including chemical composition,[98] mechanical stiffness,[99] and topography.[100] While our biologically engineered matrix resembles vascular wall tissue in all three respects, it is not a chemically defined synthetic matrix and its composition, being cell-produced, is complex. In a previous study seeding ECs onto the lumen of similarly prepared TEVG, we observed faint luminal staining for fibronectin[85] that may play an important role in ASC adhesion and mechanotransduction, even though the bulk matrix is predominantly collagen.

The similarities and differences of BM-MSC and ASC have been reviewed[101] and remained the subject of investigation, but this study further confirms the potential of the more abundant ASCs, with appropriate preconditioning, as a clinically attractive source of autologous cells that can be used to confer hemocompatibility to TEVG and other implants into the cardiovascular system. A central question that remains, however, is the extent of preconditioning necessary to ensure further EC differentiation, or at least maintenance of antithrombogenicity, post implantation.

2.6 Acknowledgments

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2.7 Figures

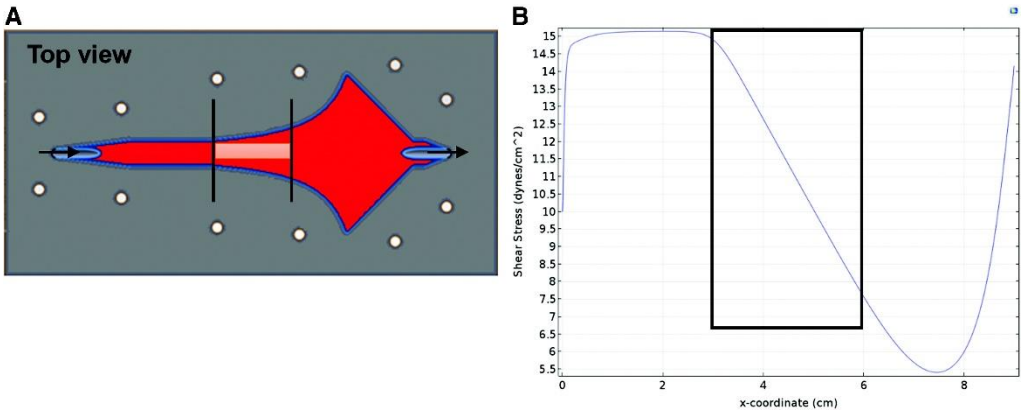

Figure 2-1. Design of linear SS chamber.

Top view (A) of linear SS chamber with (B) an inlet constant laminar SS of 15 dyn/cm^2 (x = 3 cm) that linearly decreases to 7.5 dyn/cm^2. Flow region is colored red, tissue is centered (orange), and flow direction is from left to right (black arrows). SS, shear stress.

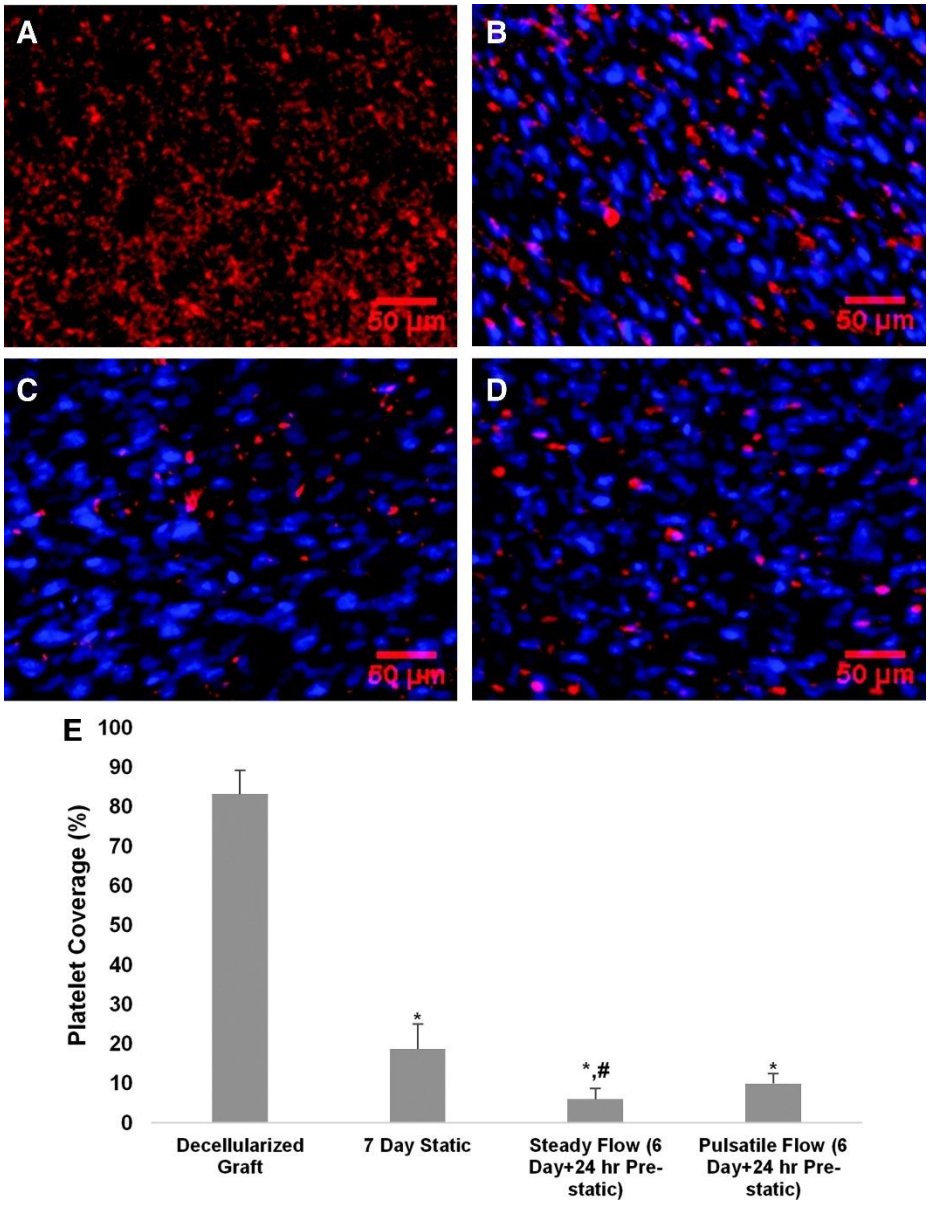
A
50 µm
B
50 µm
C
50 µm
D
50 µm
E
Platelet Coverage (%)
100
90
80
70
60
50
40
30
20
10
0
*
*,#
*
Decellularized Graft
7 Day Static
Steady Flow (6 Day+24 hr Pre-static)
Pulsatile Flow (6 Day+24 hr Pre-static)

Figure 2-2. Reduced platelet binding of TEVG seeded with shear conditioned ASC.

(A) Decellularized graft without ASC (positive control). (B) 7 day static. (C) Constant flow (6 day +24 h pre-static). (D) Pulsatile flow (6 day +24 h pre-static). (E) Quantified platelet coverage as % of graft surface area from ImageJ analysis. Blue = nuclei, red = platelets (glycoprotein IIb/IIIa). *$p < 0.001$ vs. decellularized graft (positive control). $^{\#}p < 0.05$ vs. 7 day static ($n = 4$). Scale bar = 50 μm. ASC, adipose-derived stem cell; TEVG, tissue-engineered vascular grafts.

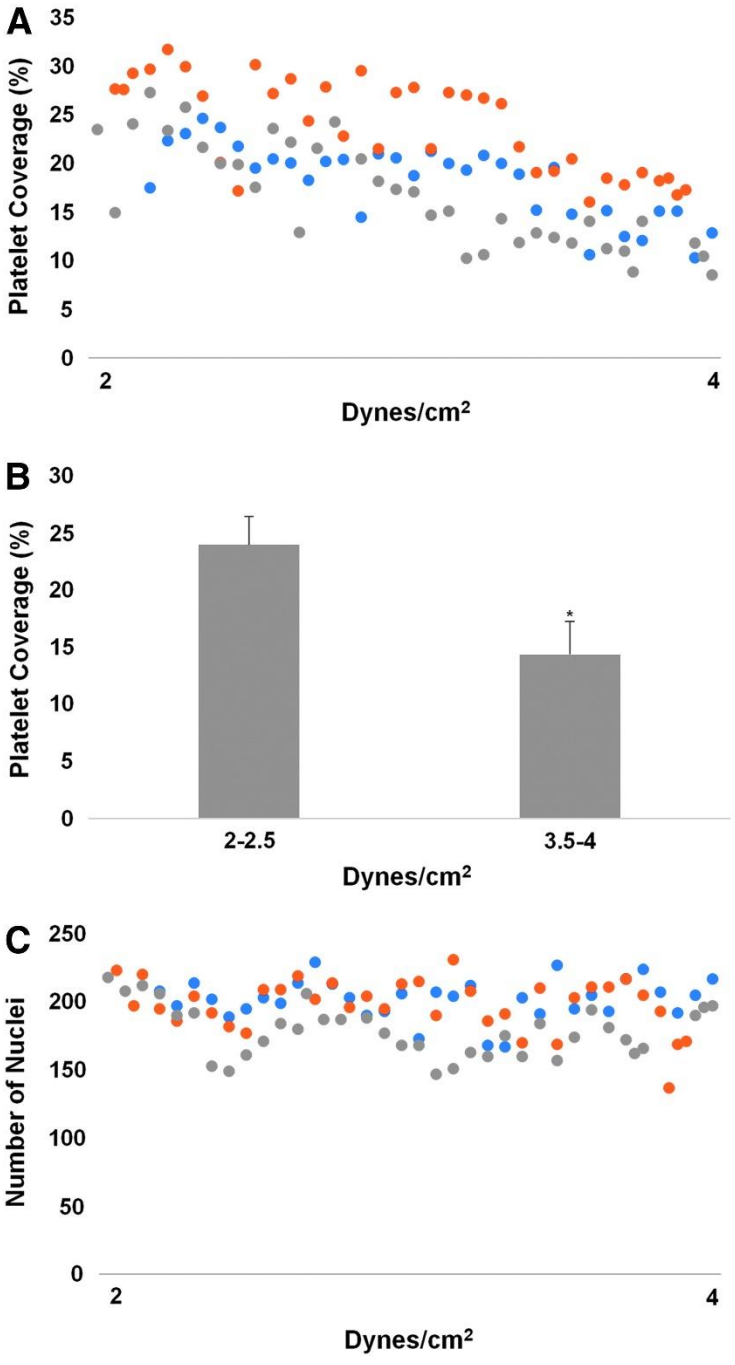
A
Platelet Coverage (%)
35
30
25
20
15
10
5
0
2
4
Dynes/cm²
B
Platelet Coverage (%)
30
25
20
15
10
5
0
*
2-2.5
3.5-4
Dynes/cm²
C
Number of Nuclei
250
200
150
100
50
0
2
4
Dynes/cm²

Figure 2-3. Platelet binding at low constant SS.

(A) Platelet coverage along the length of the chamber from inlet 4 dyn/cm^2 to outlet 2 dyn/cm^2. Data from three independent experiments are color coded (n = 3). (B) Average platelet coverage showing a decrease in the range 2–2.5 dyn/cm^2 vs. 3.5–4 dyn/cm^2 (the average value for each sample in both ranges of SS was determined for each experiment, and the averages for the three experiments are plotted). (C) Cell coverage quantified as number of nuclei for the same experiments showing no loss of cells at the higher SS locations. *$p < 0.001$ vs. 2–2.5 dyn/cm^2.

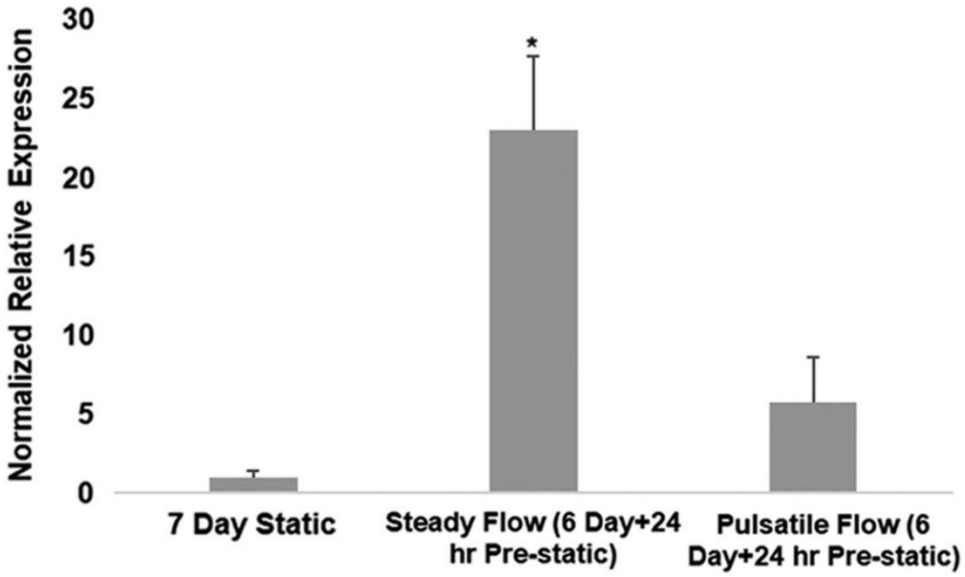

Figure 2-4. Nitric oxide production of shear conditioned ASC.

*$p < 0.001$ vs. 7 day static and pulsatile flow ($n = 4$).

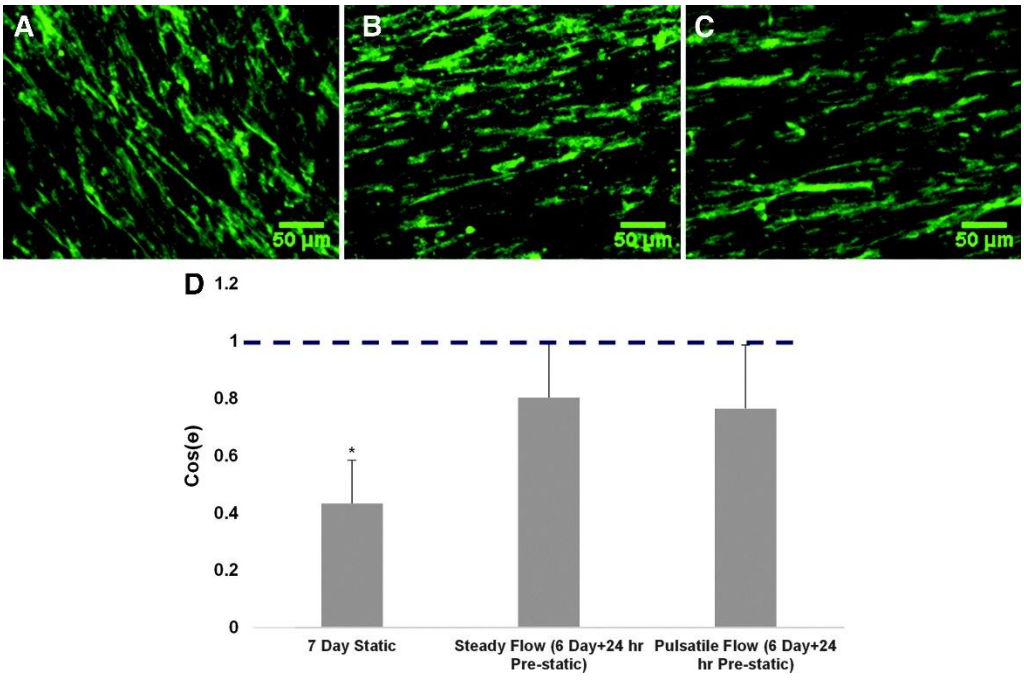

Figure 2-5. Alignment (F-actin) of ASC in response to SS.

(A) 7 day static. (B) Constant flow (15 dyn/cm^2). (C) Pulsatile flow (22 dyn/cm^2 max; Supplementary Fig. S5). (D) Quantified ASC alignment with respect to SS direction (n = 4). *$p < 0.01$ vs. theoretical value of 1 for perfect coalignment with flow. Scale bar = 50 µm.

Chapter 3. Effects of circumferential cyclic stretching versus pulsatile fluid flow in directing adipose-derived stem cells into endothelial cells

3.1 Synopsis

Adipose-derived stem cells (ASCs) can differentiate into endothelial cells through various shear stress (SS) with or without chemical treatment and acquire non-thrombogenic property. We further explored the additional influence of circumferential cyclic stretching (CS) to SS from pulsatile fluid flow. ASCs were seeded onto our biologically engineered matrix and conditioned with CS vs CS+SS for 6 days at 5% strain and 0.6 Hz through our custom bioreactor. To separate CS effect from SS, the lumen of tubular grafts was everted (inside-out lumen) to maintain same luminal matrix when seeding ASC for CS and CS+SS groups. After exposing ASC to CS and CS+SS for 6 days, platelet binding was similar in CS and CS+SS. No endothelial and smooth muscle cells were observed via immunostaining but CS and CS+SS increased nitric oxide production (5-fold) in ASC-seeded grafts compared to static group. ASC aligned perpendicularly to CS direction but parallel to flow direction, indicating either CS and/or SS was sufficient to orient ASC away from circumferential aligned matrix. These results demonstrate no additional effect of CS in addition to SS.

3.2 Introduction

Cardiovascular disease is currently the leading cause of death in the US, accounting for 1 of every 3 mortalities in 2017.[1] Synthetic substitutes (e.g. Dacron or Teflon) show success in large-diameter bypass grafts, such as in the aorta or large peripheral arteries (>6 mm diameter),[102] but they are not suitable for replacing small-diameter vessels due to thrombosis or aneurysm.[103,104] Although autologous arteries and veins are the gold standard treatment for small-caliber bypass grafts,[105] their quantity is limited when reoperation is required or about one third of the patients do not have suitable bypass vessels.[12]

Tissue engineering can provide alternative small-diameter conduits to replace limited quantity of autologous grafts and mimic similar mechanical and compliant properties of native blood vessels.[15] Fabrication of hemocompatible-engineered bypass grafts requires lining the lumen with endothelial cells (ECs), but harvesting ECs in sufficient quantity without sacrificing blood vessels is still a challenge. Mesenchymal stem cells (MSCs), especially adipose-derived stem cells (ASCs), can be harvested noninvasively in large quantity despite patient's cardiovascular history.[48]

Differentiation of ASCs to ECs using biochemical treatment, such as vascular endothelial growth factor (VEGF), has been well investigated.[37-39] Biochemical stimuli can also play a key role in elevating endothelial-specific genes in ASCs. ECs lining the lumen of the blood vessels are exposed to pulsatile blood flow, composed of shear stress (SS) and circumferential cyclic stretch (CC) simultaneously. ECs are critical in maintaining a hemocompatible layer to thrombosis and regulating physiological control

of vascular permeability, wound healing and inflammation, which are directly influenced by blood flow through endothelial mechanotransduction.[106] In fact, hemodynamic SS affects ECs phenotype, orientation and homeostasis.

Shear stress resulted from blood flow in human small-diameter arteries ranges between 10 to 20 dyn/cm^2.[107] Due to the influence of SS on ECs, studies have replicated SS *in vitro* to investigate its effects on differentiation of adult stem cells towards ECs, as well as applying the reported SS range found in arteries. Zhang et al. induced human ASCs with combined orbital SS of 12 dyn/cm^2 (maximum at the periphery of the wells) and endothelial growth factors and found an increase in protein and mRNA levels of CD31 and vWF, as well as anti-thrombogenic markers (nitric oxide production and tissue plasminogen activator).[48] Colazzo et al. also used orbital SS of 12 dyn/cm^2 combined with VEGF and showed endothelial phenotype (CD31, FLK-1 and VE-cadherin) in differentiated human ASCs.[84] Arterial physiological SS level can induce endothelial-specific markers in ASCs and conditions ASCs towards non-thrombogenic phenotype. La et al. demonstrated ASCs exposed to SS of 7.5 to 15 dyn/cm^2 had low platelet binding compared to static culture.[108]

The application of combined SS and circumferential CS on differentiating ASC to ECs has been less explored even though ECs are constantly exposed to both SS and CS simultaneously. Shojaei et al. compared the effects of cyclic stretch (10% strain), cyclic SS (0-2.5 dynes/cm^2) and simultaneous cyclic SS+CS on human ASCs. All loadings elevated mRNA levels of VE-cadherin, but CS reduced the expression levels of Flk-1 and vWF. Cyclic SS increased both levels of Flk-1 and vWF while cyclic SS+CS had

minimal effects.[57] Kim et al. found that combined SS and circumferential CS upregulated endothelial mRNA and protein markers in human bone marrow-MSCs seeded onto electrospun poly(L-lactide-co-e-caprolactone) tubular scaffolds (5 mm diameter) and detected low levels of smooth muscle cells (SMCs) markers.[49] It is still unclear whether SS and CS have synergistic effects on endothelial differentiation. Previous studies did not separate the influence of circumferential CS platform alone from pulsatile flow compared to SS+CS on ASCs seeded onto biologically-extracellular matrix substrate with circumferential alignment emulating native arteries.

In this study, we compared the effects of circumferential CS versus simultaneous SS and circumferential CS on human ASCs seeded onto our tissue engineered vascular grafts (TEVG, 4mm diameter), which had robust mechanics and success with ECs retention *in vitro*[88] and *in vivo*.[85] Our custom bioreactor was used to apply both the CS and SS at 8 dyn/cm^2 (5% strain and 0.6 Hz).[87] The lumen of the grafts were everted to seed ASC onto the same luminal matrix as CS+SS group while separate the effect of CS from SS. We also investigated platelet binding and endothelial markers as well as SMC markers.

3.3 Materials and methods

3.3.1 ASC culture

Human ASCs purchased from Lonza were cultured (5000 cells/cm^2) and expanded in ADSC-GM medium (Lonza), 100 U/mL penicillin, and 100 ug/mL streptomycin at 37°C, 5% CO_2. ASCs were fed every 3 to 4 days until confluency (~90%) and used at passage 5.

3.3.2 TEVG fabrication

Mixture of fibrinogen (4 mg/mL) in HEPES-buffered saline (20 mM) and human dermal fibroblasts (1M/mL) was added to thrombin (1.1 U/mL) and calcium chloride (5.0 mM).[87] The suspension was injected into a tubular glass mandrel (4mm-inner diameter) pretreated with 5% Pluronic F-127. Scaffolds were cultured statically for 2 to 3 weeks in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, EMD Millipore), 100 U/mL penicillin, 100 ug/mL streptomycin, 2 ug/mL insulin, and 50 ug/mL ascorbic acid. Medium was changed 3 times per week. After static culture, the grafts were stretched at 0.5 Hz pulse frequency for 5 to 7 weeks in a custom flow bioreactor.

3.3.3 TEVG decellularization

The grafts were rinsed with PBS for 10 min, 1% sodium dodecyl sulfate (SDS, Sigma) for 6 hr and 1% Triton X-100 (Sigma) for 30 min on a gentle shaker.[50] After 1 week of PBS rinses (4°C), they were treated with deoxyribonuclease enzyme (DNase, Worthington Biochemical) in DMEM/10% FBS at 37°C, 5% CO_2 overnight. Additional PBS rinses of the grafts to remove remaining traces of SDS/Triton/DNAase/ cellular components before storing in PBS (4°C) until use.

3.3.4 Circumferential cyclic stretching

To mimic physiological circumferential stretching (CS), tubular scaffolds were mounted in a custom cell-seeding apparatus overnight at 37°C. The grafts were everted to maintain CS testing of cells seeded on the luminal surface in comparison with SS+CS group. Inside-out lumen was treated with 50 ug/mL fibronectin (Sigma, F1141). ASCs

were seeded as a monolayer onto the outer surface (inside-out lumen). The grafts were rotated at 0.3 rpm for 1.5 hr to allow for cell settling and adhesion. After 1.5 hr, they were cultured statically for 48 hr in endothelial growth medium (EGM-2, Lonza) before exposure to circumferential CS (5% strain and 0.6 Hz) for 6 days (Figure 3-1). Our custom-flow bioreactor was used to apply circumferential CS induced by a reciprocating syringe pump. Since ASCs were seeded onto the outside surface of the tubes after their eversion, they were not exposed to SS on the inside (luminal) surface of the tube, yet the CS cells were seeded onto the same matrix as the CS+SS cells to eliminate any confounding effects of matrix-dependent responses due to any heterogeneity in the remodeled fibrin matrix.

3.3.5 Combined shear stress and cyclic stretching

To study the effects of both SS and CS on ASCs compared to circumferential CS alone, ASCs seeded onto lumens of TEVG were placed onto a cell roller rotating at 0.3 rpm for 1.5 hr followed by 48 hr of static pre-incubation in endothelial cell culture medium. It was mounted in the same custom-flow bioreactor for 6 days to subject the cells to pulsatile flow with CS (Figure 3-1). A calibrated flow meter was used to measure the flow rate in the pulsatile bioreactor system, which correlates to the targeted SS level (8 dyn/cm^2) through a mathematical expression for Hagen-Poiseuille low tube flow (low Womersley number limit).

3.3.6 Platelet adhesion assay

Human platelets (Red Cross) pellets were isolated from centrifugation at 1300g for 10 min. Platelets were then washed with ‘acid citrate dextrose’ buffer (100mM

trisodium citrate, 10 mM citric acid and 136 mM glucose, pH 6.5) and spun down at 1300g for 10 min. Pellets were suspended in Tyrode's buffer (Boston BioProducts, C-4110). Tissue samples were incubated with platelets suspended in Tyrode's buffer for 2 hr at 37°C. After 2 hr, they were rinsed with Tyrode's buffer 2 to 3 times and fixed with 1% paraformaldehyde for 30 min at 4°C.

3.3.7 Immunostaining

Tissue samples were stained for CD41 for glycoprotein IIB/IIIA (Abcam, ab11024, 1:400), HSPG2 (Abcam, ab26265, 1:100), VE-cadherin (Abcam, ab33168, 1:200), CD31 (Dako, M082301-2, 1:40), VEGFR2 (Abcam, ab39638, 1:200), aSMA (Sigma, A7811, 1:800) and calponin (Abcam, ab110128, 1:100).

After fixing, samples were subjected to blocking with 5% normal donkey serum and permeabilized by 0.1% Triton X-100 (Sigma). They were stained with primary antibodies (overnight at 4°C), secondary antibodies (cy3- or Cy5-conjugated, Jackson Immunoresearch, 1:200) and Alexa Fluor 488 phalloidin (Life Technologies, 1:400). Nuclei were visualized with Hoechst 33342 (Invitrogen, H3570, 1:10000).

3.3.8 Nitric oxide assay

Medium samples from each group were stored at -80°C. Using a fluorometric assay kit (Abcam, ab65327), nitrite in the samples were converted to nitrate during 3 hr incubation. Enhancer, DAN probe, and sodium hydroxide were added to the converted nitrate and was measured at 360/450 nm (excitation/emission).

3.3.9 Alignment of ASC

Cell alignment on the matrix was evaluated post-flow by phalloidin staining and

analyzed using an ImageJ orientation plugin[89] to determine the orientation angle relative to the flow (SS) direction.

3.3.10 Statistical analysis

Experiments were repeated at least 3 times, represented by "n", and data are expressed as mean ± standard deviation. One-way ANOVA was used to calculate statistical significances for multiple groups, and a statistic value of $p<0.05$ was considered statistically significant.

3.4 Results

3.4.1 Platelet binding in ASC conditioned with CS and CS+SS

ASCs seeded onto the lumen of TEVG were cultured statically for 2 days and mounted onto CS and CS+SS custom bioreactors for 6 days at 5% strain and 0.6 Hz. Figure 3-2 shows static samples incubated with platelets had the highest platelet binding compared to CS and CS+SS group. CS and CS+SS had no significant difference in platelet binding.

3.4.2 Nitric oxide production

To assess non-thrombogenic phenotype of differentiated ASC, NO was measured from collected medium samples. Both CS and CS+SS had 5-fold increase in NO production compared static group, but there was no difference in produced NO between CS and CS+SS (Figure 3-3).

3.4.3 Endothelial cell and smooth muscle cell markers

To investigate evidence of endothelial differentiation of ASCs conditioned with CS and simultaneous CS+SS, EC markers (e.g. HSPG2, VE-cadherin, CD31, and

VEGFR2) were evaluated via immunostaining. Figure 3-4 shows that both CS and simultaneous CS+SS groups did not express any EC markers. ASC has been shown to be capable of differentiation into smooth muscle cells with biomechanical forces, specifically CS, so SMC markers (e.g. αSMA and calponin) expression was also assessed in addition to EC markers. It was found that ASC seeded onto matrix cultured statically for 2 days or 8 days did not express any SMC markers. CS or CS+SS also did not upregulate expression of aSMA and calponin (Figure 3-5).

3.4.4 ASC alignment in response to bioreactor conditioning

F-actin stained stress fibers in ASC were analyzed as $<\cos\theta>$ to measure ASC orientation, Figure 3-6. Given the predominate fluid flow is inside the tube, the direction of CS is perpendicular (circumferential) to the direction of SS (axial) for CS+SS cells, whereas it is simply a matter of circumferential stretching for CS cells (minimal shear stress on the outside surface of the tube.[109] ASC cultured statically aligned circumferentially (data not shown). ASC conditioned with CS aligned away from the direction of stretching ($<\cos\theta=0.94>$) whereas ASC conditioned with CS+SS aligned towards the direction of fluid flow ($<\cos\theta=0.90>$).

3.5 Discussion

In this study, we investigated the differentiation of ASC-seeded vascular grafts towards EC using circumferential CS and simultaneous CS+SS, resulted from pulsatile fluid flow. Previous studies have focused on using chemical factors and SS as key forces to stimulate stem cells towards vascular EC. Other biomechanical forces, such as circumferential CS and CS+SS, are less explored for endothelial differentiation. We

applied our custom bioreactor that allows separation of circumferential CS from SS in flow and combined CS+SS to hASC cultured onto our biologically-engineered matrix. Our SS study in Chapter 2 explored 7.5-15 dyn/cm^2 onto ASC and found low platelet binding, hence we chose 8 dyn/cm^2 (0.6 Hz and 5 % strain) for our CS vs CS+SS study.

To evaluate the ability of ASC capable of differentiation into EC, endothelial markers and non-thrombogenic property of differentiated ASC were evaluated. While SS and additional effect of CS (3% and 5% strain at 2 Hz)[49] have been reported to increase levels of EC markers, we did not observe any EC markers (e.g. HSPG2, CD31 or VEGFR2) in either CS or CS+SS at 5% strain and 1 Hz, but we did observe a similar increase in NO production compared to the static control group.

In addition to no observation of EC markers in ASC, upregulation of SMC markers was also not present. Both CS and CS+SS conditionings for 6 days did not yield αSMA and calponin via immunostaining. Wang et al.[110] applied circumferential 5% strain to hASC seeded onto polyglycolic mesh (pre-static incubation with chemical factors for 7 days) for 8 weeks and found an increase in expression of αSMA, calponin and smooth muscle myosin heavy chain (SM-MHC). Mechanical strain at 12%, 1 Hz for 96 hr demonstrated by Girao-Silva et al. did not elevate levels of SMC markers in ASC.[111] This could be due to difference in stretching parameters and duration, in combination with different chemical factors. Although our results showed no signs of SMC markers, longer stretching period can potentially lead to SMC derived from ASC.

Endothelium is continuously exposed to pulsatile blood flow, composed of cyclic circumferential strain and SS. It is highly responsive to mechanical stimuli, which aligns

perpendicular to direction of uniaxial stretch[112]and parallel to direction of flow.[113] Our study shows when cyclic circumferential strain was applied for 6 days, ASC oriented away from strain direction but along with flow direction. Stress avoidance and/or SS-induced alignment were sufficient to overcome any contact guidance response to the circumferential-aligned matrix.

In summary, this study has demonstrated no additional benefit or adverse consequence of CS in addition to SS in differentiating ASC towards EC phenotype at 5% strain and 0.6 Hz for 6 days. CS and CS+SS yielded similar results in platelet binding, NO production, and alignment.

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3.7 Figures

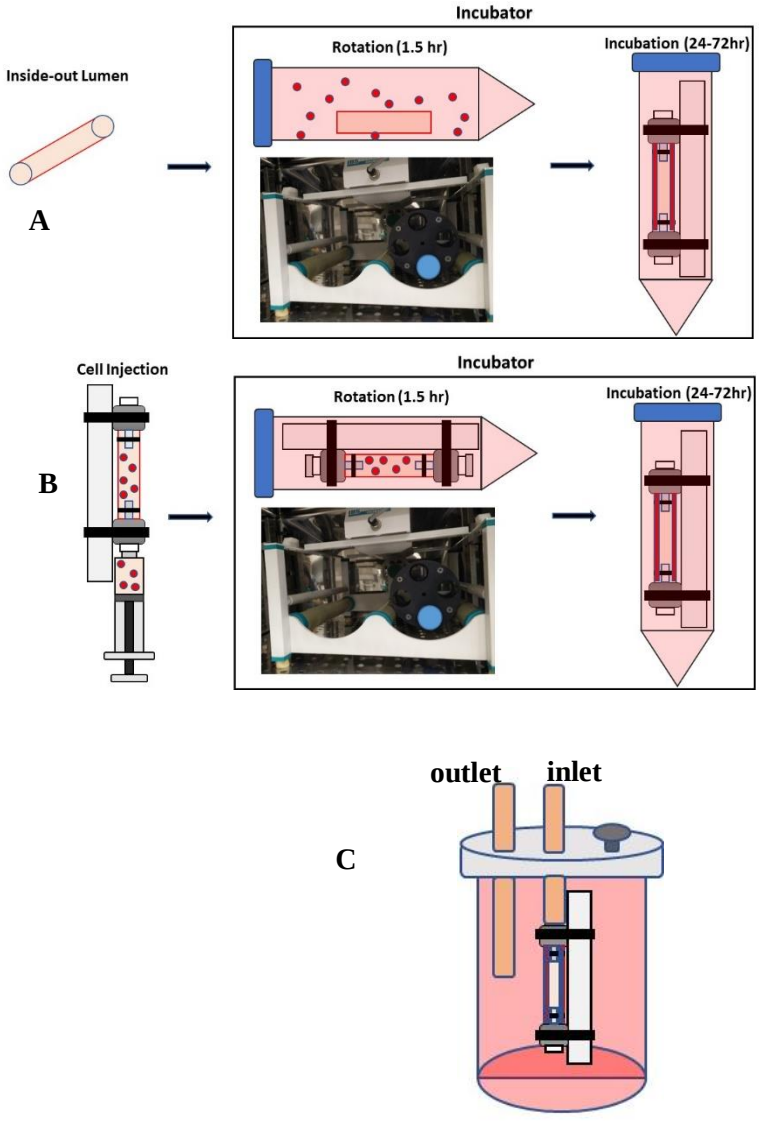
Incubator
Rotation (1.5 hr)
Incubation (24-72hr)
Inside-out Lumen
A
Cell Injection
B
Incubator
Rotation (1.5 hr)
Incubation (24-72hr)
outlet
inlet
C

Figure 3-1. Experimental set-up of CS and CS+SS.

(A) ASCs seeded onto everted luminal surface to separate CS from SS. (B) ASCs seeded onto luminal surface (not everted) for CS+SS. (C) Custom bioreactor for CS and CS+SS groups.

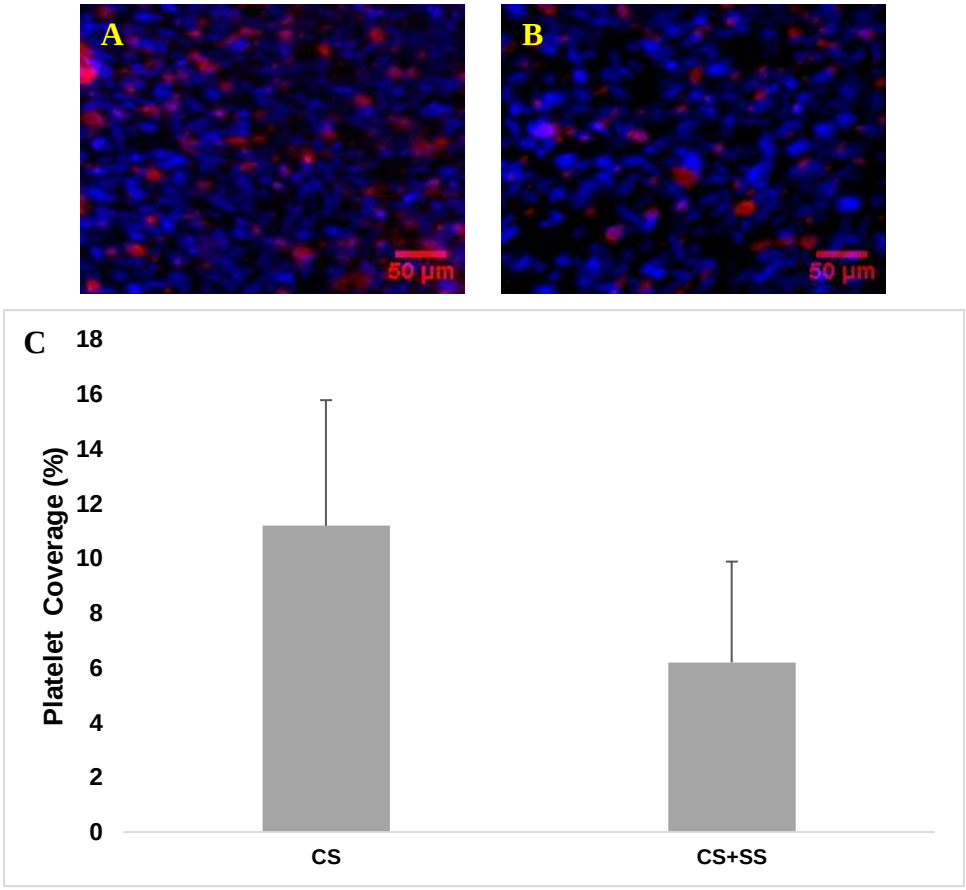

Figure 3-2. *In vitro* thrombogenicity assessment of conditioned ASC. Platelet binding of ASC in (A) CS and (B) CS+SS. (C) Quantified platelet coverage as % of graft surface area from ImageJ analysis Blue=nuclei, red=platelets (glycoprotein IIb/IIIa). Scale bar = 50 um. n=4.

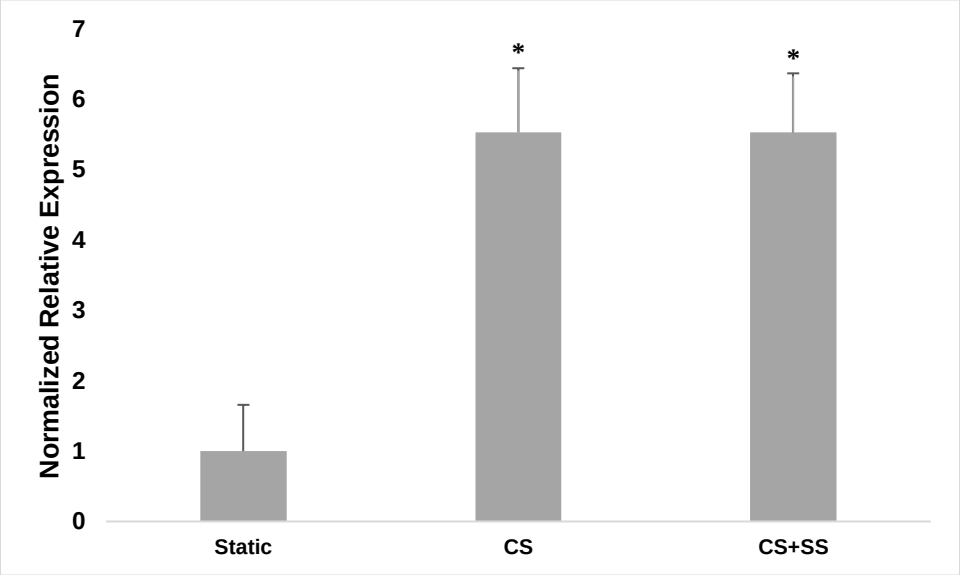

Figure 3-3. NO production in stimulated ASC.

*=p<0.01 vs. static (n=4).

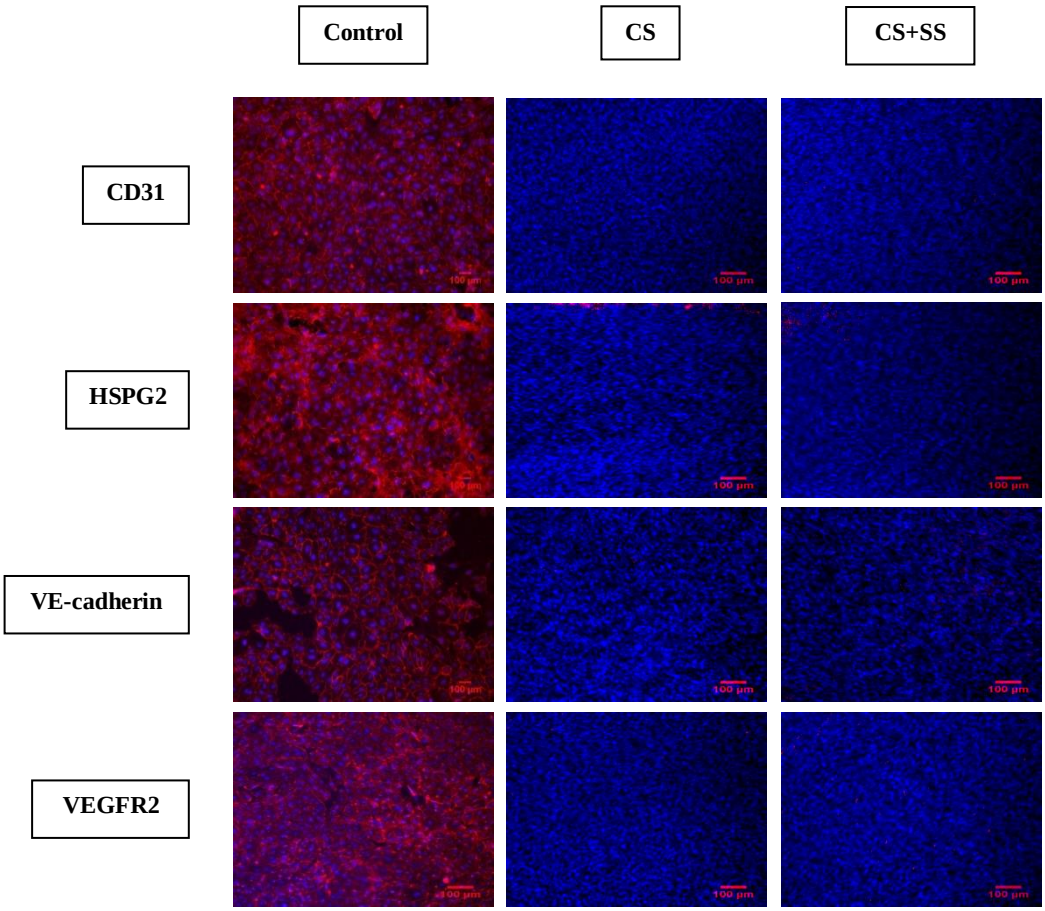

Figure 3-4. Characterization of endothelial markers in ASC conditioned with CS and CS+SS for 6 days.

Human umbilical vein endothelial cells (control). Scale bar = 100 um.

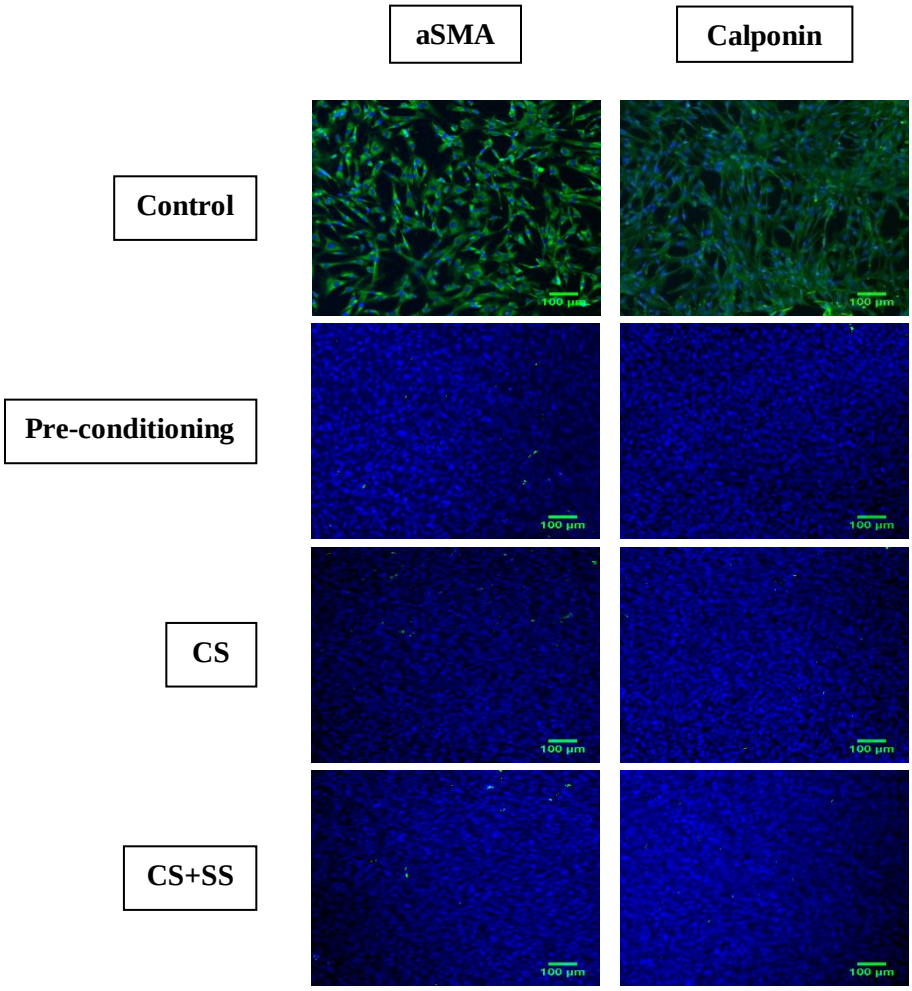

Figure 3-5: Evaluating aSMA and calponin markers in ASC conditioned without biomechanical forces (pre-conditioning for 2 days), CS and CS+SS.

Smooth muscle cells (control). Scale bar = 100 um.

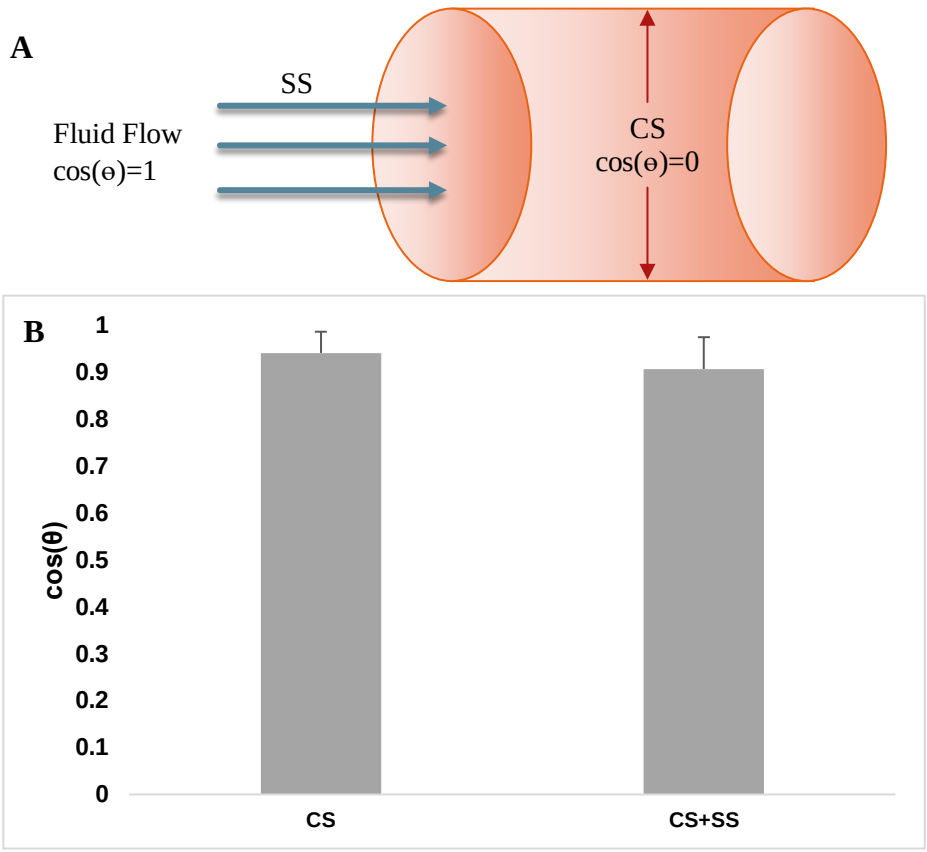

Figure 3-6: Measurement of F-actin expressed by ASC in response to fluid flow.

(A) Schematic sketch of fluid flow composed of SS and CS. (B) Quantified ASC alignment with respect to shear stress direction (n=4).

Chapter 4. Conclusions and Future Directions

4.1 Major contributions

This Ph.D. research has contributed significantly to the field of TEVG through publication in *Tissue Engineering Part A* on "Shear conditioning of adipose stem cells for reduced platelet binding to engineered vascular grafts" (Chapter 2) and posters and oral presentations at international conferences. This work has developed a custom PPFC to study a range of SS per chamber as well as investigating the influence of physical stimulations on endothelial differentiation of human ASC, isolating the effects of laminar SS, pulsatile SS, and CS along with the physiologically relevant combination of pulsatile CS and SS. These major contributions provide further insights towards the development of creating a hemocompatible TEVG.

4.1.1 Creation of linear flow chamber for applying uniform range of shear stress

Previous works applied SS via orbital shaker to induce ASC into EC, creating a lack of uniform SS and inaccurate measurement of SS magnitude. Chapter 2 details the adaptation of a custom parallel-plate flow chamber designed by Usami et al. (1993), which allows a range of SS levels to be studied simultaneously (since SS varies linearly with position along the long axis of the chamber). It removes the constraint of one SS magnitude per chamber to establish cost-effective experimental trials. It also can be used to assess either steady laminar or pulsatile SS that is lacked in orbital shaker. In addition, existing PPFC typically allows study of cells seeded onto non-matrix. This chamber incorporates the differentiation of ASC seeded onto our TEVG, mimicking the ECM of native tissues.

4.1.2 Establishment of non-thrombogenic properties of ASC conditioned with biomechanical forces

Prior to this study, differentiation of ASC to EC was mostly characterized for EC markers via *in vitro* measurement of mRNA and protein levels, but less focus on evaluation of thrombogenicity of differentiated ASCs. Chapter 2 and 3 apply *in vitro* thrombogenicity assay (e.g. platelet adhesion) to induce ASCs. It was determined that average constant linear SS yielded lowest platelet binding in ASC compared to pulsatile SS and static culture groups, consistent with highest NO production. However, no platelet binding dependence on the magnitude of SS was found in the range of 7-15 dyne/cm^2, but a roughly linear graded reduction in platelet binding was found over the range of 2-4 dyne/cm^2.

Chapter 3 describes a method to separate the influence of CS from pulsatile SS exerted on ASC seeded onto the compliant TEVG (mimicking the circumferential CS resulting from pulsatile flow). The use of a custom bioreactor allows study of circumferential CS on ASC-seeded TEVG as well as combined pulsatile CS+SS inside the lumen. Results demonstrated no significant difference in platelet binding in ASC between CS and CS+SS groups as well as NO production. Immunostaining indicated no EC and SMC expression.

4.2 Future directions

Although the findings of this work show evidence of some acquired endothelial characteristics in conditioning ASC, further validation of additional EC markers and fully differentiated stage of ASC is needed to achieve successful hemocompatible grafts.

4.2.1 Determination of endothelial markers in ASC conditioned with biomechanical factors via quantification of mRNA and protein levels

Conditioning ASC with biomechanical forces upregulated NO production and yielded lower platelet binding compared to static culture. However, immunostaining showed no signs of any EC markers, indicating possible early differentiation of ASC into EC or other cell type (e.g. SMC). Probing for signs of mRNA and proteins levels can be achieved through qPCR and Western Blot to confirm differentiated ASCs towards mature EC. Expression of other cell type, specifically SMC, is also needed to be characterized for each of the biomechanical forces. Longer conditioning time can be used to apply biomechanical forces onto ASCs to induce differentiation towards ECs.

4.2.2 Evaluation of non-thrombogenic and inflammatory markers

Platelet binding assay showed biomechanical conditioned ASC-seeded grafts acquired antithrombogenicity compared to non-conditioned ASC-seeded grafts, but investigation of other non-thrombogenic markers is needed to ensure further non-thrombogenic property of conditioned ASC. Such markers can be secretion of tPA that is responsible for fibrinolysis to prevent blood clot. Even if fully differentiated ASC into EC is achieved, potential inflammation of these cells could be resulted from *in vitro* biomechanical conditioning. Inflammation of an endothelium could result in disruption of hemocompatibility of the seeded grafts post implant. Important markers playing a role in inflammation process, such as ICAM-1 and VCAM-1, can be evaluated.

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